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(54) Title: PROCESS FOR THE INTRODUCTION OF EXOGENOUS DNA IN SOMATIC AND GERM ANIMAL CELLS

(57) Abstract

A process is described for the introduction of exogenous DNA into somatic and germ animal cells: the DNA, exogenous or modified according to known techniques of recombinant DNA, is introduced into the animal spermatozoa which are to be modified and said spermatozoa are employed for egg fertilization according to usual artificial fertilization techniques.

PROCESS FOR THE INTRODUCTION OF EXOGENOUS DNA IN SOMATIC AND GERM ANIMAL CELLS

1. Field of the invention

The present invention refers to a process for the introduction of exogenous DNA into somatic and germ animal cells.

In particular, the process consists in introducing DNA, exogenous or modified according to known techniques of recombinant DNA, into the spermatozoa of the animal which is to be modified and in employing said spermatozoa for egg fertilization according to known techniques for artificial fertilization.

2.Prior technique

The creation of transgenic animals, that is of animals in which are permanently integrated genetic informations extraneous to their own genomes and deriving from other genetic systems, has been and still is an objective of primary importance for the study of genetic regulation, both for chemical and therapeutical ends and for breeding domestic mammals, fish, echinoderma and amphibia.

It is possible in fact to create animals with particular advantageous characteristics, such as e.g. rate of growth or resistance to certain diseases in the case of animals for breeding, or, viceversa, predisposition to certain diseases in the case of animals utilized for experimenting new drugs. The first attempts at obtaining transgenic animals go back to the middle of the seventies. Those attempts were chiefly based on the manipulation of mice embryos or of cultured cells and on the direct DNA (e.g. SV 40)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
T	Cell, volume 59, 20 October 1989, Cell Press, R.L. Brinster et al.: "No simple solution for making transgenic mice", pages 239-241 see the whole letter and reply	1-6

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high rate of abortions, the high rat of mosaicism in the obtained animals and the marked sterility of the same.

3. The technical problem

The fundamental technical problem which is solved by the present invention is the introduction of DNA,treated according to known techniques of recombinant DNA,into the cellules of an animal pertaining to an animal species which does not actually posses the typical sequences of the introduced exogenous DNA, with the result that the genetic informations contained in said recombinant DNA is permanently integrated in the genomes of the treated individual and may therefore be transmitted to the successive progeny of the individual.

D. Detailed description of the invention

The process for the introduction of cloned DNA into the cells of a different species according to the present invention is based on an experimental observation, namely the surprising easiness with which molecules, even if of large dimensions, succeed in penetrating into the spermatozoa head.

This property, typical of spermatozoa both of mammals and of other animal species, was utilized to modify the spermatozoa, intorducing in them the cloned DNA to be transferred.

With the modified spermatozoa, the corresponding oocytes are then fertilized by means of the artificial fertilization techniques employed with unmodified spermatozoa.

According to a fundamental characteristic of the present invention,

5. Process according to claim 2, characterized in that the oocytes to be fertilized are added to the aqueous incubated spermatozoa suspension and the mixture is incubated for 5 to 10 hours at a temperature of from 20° to 37°C, in air containing more than 5% to 10% of carbon dioxide.

6. Process according to claim 5, characterized in that the embrious obtained from the fertilized oocytes, as described, when reaching the development stage of two cells are surgically transferred into the oviducts of pseudogravid females.

We have employed this animal because all the laboratory technique for its "in vitro" fertilization and for the study of the integration and expression of its genes are amply reported in the scientific literature.

As exogenous DNA we used p SV2 CAT, Polyoma and the human growth gene, because their restriction maps are described in literature and comprise base sequences which are not naturally present in mouse genome.

The identification of these sequences in the "positive" mouse, that is in the mouse obtained from the egg fertilized with the treated spermatozoa, allows to ascertain without the shadow of a doubt that the cloned DNA was actually introduced into the treated spermatozoa and through these into the fertilized eggs and therefore integrated into the genome of the resulting transgenic individuals.

a) preparations of the spermatozoa.

A spermatozoa suspension was prepared by pressing the epididymis of a male mouse into 1 ml PM buffer (prepared as described by D.G. Whittingam. Culture of Mouse - ove - (1971) - J Reprod. Fert. Supp. 14, p.7-21).

The spermatozoa suspension was centrifuged so to separate the spermatozoa which were again suspendend in 1 ml of buffer.

The above treatment was repeated 5 times so to "wash" the spermatozoa by assuring the complete elimination of seminal liquor.

The buffer was modified eliminating sodium lactate, penicillin and steptomycin, substituting monosodiumphosphate by 0.15 mM

place in that particular region, it is believed that also the exogenous DNA is trasferred at the same time and in the same way as the DNA proper of the species. This mechanism might explain the surprising efficiency of the process according to the invention.

hours at a temperatur of from 20° to 37°C, in air containing more than 5% to 10% of carbon dioxide. At the end of said period, the eggs are washed with M16 buffer (prepared as described by Whittingam -see point a) supra) and left for an entire night in 50 µl of the same buffer.

After 24 hours the embryos are surgically transferred, at the stag of two cellules, into the oviducts of pseudogravid females.

The offsprings deriving from these implants, at the age of three weeks, are amputated of a terminal tail fragment, from which the DNA is extracted which is analyzed with the aid of the "Southern blot" described in the book "Molecular Cloning": A Laboratory Manual" by T. Maniatis et al. - C.S.M., New York 1984.

This analysis allows to identify "positive" individuals, that is those whose genome posses, integrated or in episomic form, one or more copies of the same cloned DNA introduced into the starting spermatozoa.

The yield of "positive"individuals obtained following the process of the present invention is always higher than 30% up to 70%, and, what is more, no sterile individuals are found among them.

The successive genetic characterization of the positive animals is carried out with the two analysis methods of restriction and sequence.

The analysis of the genome DNA of positive mice was carried out according to two methods:

Restriction Analysis

DNA restricti n analysis by means f specific restriction enzymes which allow the subdivision of genome DNA into fragments. The obtained fragments are then fractionated by electrophoresis and transferred onto a nitrocellulose filter according to the known Southern blot technique. The filter is then treated with the probe specific for the initial cloned DNA employed for transforming the spermatozoa, made radioactive with P^{32} and exposed on a x-ray sensitive film.

The film will be exposed in correspondence with the sites where the radioactive probe was bound to the filter, that is in correspondence with each radioactive DNA bond, leaving a signal for each DNA fragment.

The presence of one or more signals, their number and the dimensions of the DNA fragments which they represent allow to conclude that sequences exist which are analogous to the positive mouse genome probe, and to determine a restriction map.

The analogy between this map and the one of the cloned DNA introduced into the spermatozoa from which the "positive" mouse originates proves that the original clone sequences are integrated into the transgenic mouse genome.

- Sequence analysis

To analize the sequence of the bases constituing the "positive" mouse genome, equal amounts (15 µg) of genome DNA of a positive mouse and of the pUC13 plasmid resistant to ampicillin were restricted with the restriction EcoRI enzyme.